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Nitrogen limited biobarriers remove atrazine from contaminated water: Laboratory studies

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ABSTRACT

Atrazine is one of the most frequently used herbicides. This usage coupled with its mobility and recalcitrant nature in deeper soils and aquifers makes it a frequently encountered groundwater contaminant. We formed biobarriers in sand filled columns by coating the sand with soybean oil; after which, we inoculated the barriers with a consortium of atrazine-degrading microorganisms and evaluated the ability of the barriers to remove atrazine from a simulated groundwater containing 1 mg L^{-1} atrazine. The soybean oil provided a carbon rich and nitrogen poor substrate to the microbial consortium. Under these nitrogen-limiting conditions it was hypothesized that bacteria capable of using atrazine as a source of nitrogen would remove atrazine from the flowing water. Our hypothesis proved correct and the biobarriers were effective at removing atrazine when the nitrogen content of the influent water was low. Levels of atrazine in the biobarrier effluents declined with time and by the 24th week of the study no detectable atrazine was present (limit of detection<0.005 mg L^{-1}). Larger amounts of atrazine were also removed by the biobarriers; when biobarriers were fed 16.3 mg L^{-1} atrazine 97% was degraded. When nitrate (5 mg L^{-1} N), an alternate source of nitrogen, was added to the influent water the atrazine removal efficiency of the barriers was reduced by almost 60%. This result supports the hypothesis that atrazine was degraded as a source of nitrogen. Poisoning of the biobarriers with mercury chloride resulted in an immediate and large increase in the amount of atrazine in the barrier effluents confirming that biological activity and not abiotic factors were responsible for most of the atrazine degradation. The presence of hydroxyatrazine in the barrier effluents indicated that dehalogenation was one of the pathways of atrazine degradation. Permeable barriers might be formed in-situ by the injection of innocuous vegetable oil emulsions into an aquifer or sandy soil and used to remove atrazine from a contaminated groundwater or to protect groundwater from an atrazine spill.

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1. Introduction

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is used in agriculture to control many broadleaf and certain grass weeds in corn (*Zea mays* L.), grain sorghum [*Sorghum bicolor* (L.) Moench], and sugarcane (*Saccharum officinarum* L.). Atrazine is one of the most frequently applied pesticides (AATSE, 2002; USEPA, 2006) and spillage, application problems, runoff, and leaching associated with its usage has made it one of the most commonly detected pesticides in water in Australia, Canada, China, Germany, France, the United States and other regions (Pick et al., 1992; Seiler et al., 1992; Kolpin and Kalkhof, 1993; Guzzella et al., 1996; Dorfler et al., 1997; Garmouma et al., 1997; Kookana et al., 1998; Masse et al., 1998; Jin and Ke, 2002; Silva et al., 2004). In silt loan soils atrazine is highly mobile and in deeper soils and aquifers, highly persistent (Starr and Glotfelty, 1990; Klint et al., 1993; Assaf and Turco, 1994; Papiernik and Spalding, 1998). This has often led to groundwater contamination problems in agricultural areas

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(Gojmerac, 1996; Kolpin et al., 1997; Tappe et al., 2002; Guzzella et al., 2006). Its persistence as a groundwater contaminant is evidenced by its continued occurrence in groundwaters in Germany and Italy 10 to 20 years after its use was banned (Tappe et al., 2002; Guzzella et al., 2006). Atrazine is a suspected carcinogen and possible endocrine disrupter; in addition, it may interfere with cell proliferation (Health-Canada, 1993; Manske et al., 2004; Gammon et al., 2005). In the European Union the maximum contaminant level (MCL) for atrazine in water is 0.1 μg L^{-1} while in the United States and China the MCL is 3 μg L^{-1}. The European Union has banned its use.

Physiochemical methods, such as distillation, reverse osmosis, nanofiltration (Zhang et al., 2004), bonding to polymers (Pulko et al., 2007), and sorption onto charcoal (Li et al., 2003) or polymers (Masqué et al., 1998; Hollink et al., 2005) can effectively remove atrazine and other triazines from water and are good choices for small volumes of water containing higher amounts of atrazine. However, for groundwater applications involving very large volumes of water that contain only small amounts of atrazine these approaches become expensive because of the equipment required for the process. In addition, physiochemical processes that concentrate atrazine into a waste that must be disposed of can present a waste disposal problem.

What is clear is that inexpensive treatment methods that can protect groundwater from contamination and that can remediate already contaminated aquifers by destroying the toxicity of the atrazine molecule are needed. In-situ methods that dehalogenate atrazine are likely to be the most economic and effective methods of remediating contaminated aquifers. Costs associated with in-situ barriers are largely associated with their initial installation. In-situ barriers, once installed, often have long life-expectancies and low long-term operating costs as they utilize the natural movement of groundwater to bring the contaminant(s) into contact with the reactive zone within the barrier.

Several in-situ approaches have been employed or proposed for atrazine. Williamson et al. (2000) employed a funnel-and-gate in-situ system using charcoal to remove atrazine and other pesticides from groundwater. This approach would sequester the groundwater contaminants but would not destroy them.

In-situ methods that pump air into anaerobic aquifers or employ oxidants such as ozone and hydrogen peroxide have been proposed as possible treatment methods and used in laboratory, pilot, and full-scale field procedures to remediate a number of chlorinated organic groundwater contaminants (ITRC, 2005). In some cases these approaches have resulted in enhanced atrazine degradation (Patterson et al., 2002) and in other they have not (Arildskov et al., 2001). Key factors that may influence the success of this type of remediation include the presence or absence of an atrazine-degrading microbial population, amount and type of electron donor present, and the amount and type of nitrogen presence (Entry et al., 1993; Alvey and Crowley, 1995; Abdelhafid et al., 2000a,b; Patterson et al., 2002). Other approaches would be needed for the treatment of aerobic groundwater.

Laboratory studies suggest that zero-valent iron can reductively dehalogenate atrazine (Monson et al., 1998). In addition, phytoremediation may remove atrazine from soils (Burken and Schnoor, 1996, 1997) and from shallow aquifers (Susarla et al., 2002). All of these methods have potential as possible remediation approaches for groundwaters that are contaminated with atrazine. However, these approaches have not been fully evaluated under field conditions and, at the present time, only limited information is available on the advantages and disadvantages of these different technologies and the cost associated with their use. It is likely that different approaches will work better at different sites and that no single technology will meet all needs. Continued research is warranted.

In the mid-1990s bacteria able to catabolize atrazine were isolated (Mandelbaum et al., 1995; Radosevich et al., 1995). Subsequently, the genes and enzymes responsible for atrazine catabolism by soil bacteria were identified. Attempts have been made to utilize pure or mixed cultures of atrazine-degrading bacteria for bioremediating soils, groundwater or waste water streams contaminated with the herbicide (Assaf and Turco, 1994; Alvey and Crowley, 1996; Grigg et al., 1997; Shapir and Mandelbaum, 1997; Struthers et al., 1998; Newcombe and Crowley, 1999; Crawford et al., 2000; Strong et al., 2000; Topp, 2001). Although most of these trials have been successful, they often required high inoculum densities and frequent re-inoculation.

A promising in-situ technology that has gained wide acceptance in recent years involves the use of biobarriers that contain vegetable oil as a microbial electron donor (Hunter et al., 1997; Hunter, 2001). These barriers differ from other biological barriers in that the substrate used to stimulate subsurface microbial activity is an insoluble liquid that can be injected into the aquifer to create a stationary or nearly stationary biobarrier (Hunter, 2005). Numerous research studies and remediation projects have shown that these biobarriers can be used to remove nitrate, chlorate, perchlorate, heavy metals and a variety of chlorinated solvents from groundwater (Hunter and Follett, 1995; Hunter et al., 1997; Lee et al., 2000; Zenker et al., 2000; Lee et al., 2001; Wiedemeier et al., 2001; Hunter, 2002; Waddill et al., 2002; Hunter and Kuykendall, 2005). Vegetable oil-based biobarriers are frequently used for the in-situ remediation of groundwater that is contaminated with tetracholorethylene and trichloroethylene.

Vegetable oil, as well as a number of other potential biobarrier substrates, has a high carbon content and little or no nitrogen. Thus, a potential application for these types of substrates is to use them to create biobarriers where it is nitrogen availability that limits microbial activity. Such barriers, when inoculated with the appropriate bacteria, might be used to remove nitrogen containing contaminants from groundwater.

The objective of this investigation was to use vegetable oil to create a biobarrier where nitrogen availability limited microbial activity, to inoculate the barrier with atrazine-degrading microorganisms, and to determine if the barrier would efficiently remove the atrazine parent molecule from flowing water. An additional objective was to determine whether or not atrazine was biologically degraded within the barriers. The laboratory biobarriers serve as models of field based biobarriers and successful results would support the hypothesis that barriers containing high carbon-low nitrogen substrates might serve as useful in-situ tools for the

remediation of groundwaters that have contaminants, such as atrazine, that contain nitrogen.

2. Materials and methods

2.1. Chemicals

Analytical standards of atrazine (purity 99.2%), hydroxyatrazine (purity 96%), deisopropylatrazine (purity 99.1%), and deethylatrazine (purity 99.5%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, 53201). Atrazine incorporated into the influent buffer used in the biobarriers was AAtrex nine-0 (Syngenta, Greensboro, NC). Solvents used were HPLC grade. All other chemicals were reagent grade.

2.2. Biobarriers used in the initial study

Barriers, 25 cm long, were formed by packing two water-jacketed glass chromatography columns (Pharmacia, Uppsala, Sweden) 2.6 cm in diameter with 195 g of silica sand (Hunter, 2001) that had been coated with 2.5 g of soybean oil. After the barrier was formed 5 cm of oil-free sand (40 g) was added to the upper effluent end of the columns giving a 30-cm-long column of sand with a pore volume of 68 mL. Biobarriers were maintained at 25 °C in the dark and were supplied with a reconstituted water (Greenburg et al., 1992) pumped upwardly through the columns. The reconstituted water was stored at 4 °C and contained $CaHCO_3$, 96; $CaSO_4{\cdot}H_2O$, 60; $MgSO_4{\cdot}7H_2O$, 60; KH_2PO_4, 43.8; $(NH_4)_2SO_4$, 5; KCl, 4.0; FeEDTA, 1.8; H_3BO_3, 0.5; $MnCl_2{\cdot}4H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2{\cdot}H_2O$, 0.01; $MoCl_3$, 0.01 mg L^{-1}. Water pH was 7.4–7.8, hardness was 80–100 as mg L^{-1} $CaCO_3$, and alkalinity was 60–70 mg L^{-1} as $CaCO_3$. Flow rate was ~30 mL or ~13 cm day^{-1}. Oxygen was not removed from the influent water supplied to the biobarriers. The water was supplemented with atrazine at a concentration of 1 mg L^{-1} unless otherwise indicated. Each biobarrier was injected with a microbial inoculum described in Section 2.5. Effluents were analyzed for atrazine and hydroxyatrazine using a high-pressure liquid chromatograph (HPLC) equipped with a diode array detector (see below). At 5 weeks into the study each biobarrier received 3 mL of an atrazine-degrading microbial inoculum (see below). A schematic showing the column layout is presented in Fig. 1.

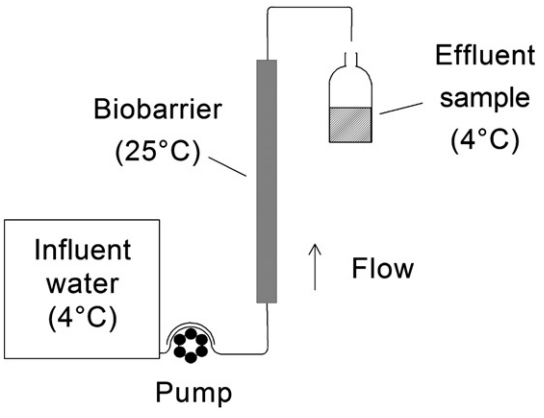

Fig. 1. Schematic of the column system.

2.3. Atrazine removal in the presence of nitrate

A second study, using the design outlined in Section 2.2, looked at the impact of nitrate-N on the degradation of atrazine. For this study the influent water was supplemented with $NaNO_3$ (5 mg L^{-1} N).

2.4. Biological vs. abiotic degradation

A third study, using the design outlined in Section 2.2, looked at the impact of mercury poisoning on the degradation of atrazine. For this study the influent water was supplemented with HgCl (0.05 mM) during the final weeks, 32 through 42, of the study. Prior to week 32 this study serves as a duplicate of the initial study.

2.5. Atrazine-degrading microbial inoculum

A soil from Wiggins, CO that had a history of atrazine use (Shaner and Henry, 2007) was extracted 1:1 (w:v) with an HM salts media (Hunter and Kuykendall, 2006) that contained 10 mg atrazine (AAtrex), 4.5 g sodium citrate, 1.3 g *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid, 1.1 g 2-(*N*-morpholino) ethanesulfonic acid, 27 mg iron-ethylenediaminetetraacetic acid, 88 mg $MgSO_4$, 13 mg $CaCl_2$, 250 mg Na_2SO_4, 125 mg Na_2HPO_4, 0.5 mg H_3BO_3, 0.1 mg $MnCl_2{\cdot}4H_2O$, 0.01 mg $CuCl_2{\cdot}H_2O$, 0.01 mg MoO_3, and 0.1 mg $ZnCl_2$ L^{-1}. The extract was centrifuged at 500 ×*g* for 5 min and 10 mL of the supernatant fluid mixed with 100 mL of fresh HM salts+atrazine media. The mixture was incubated at 30 °C and 100 rpm. After 5 days incubation heavy growth was evident and 3 mL of this bacterial culture was injected onto each biobarrier as a bacterial inoculum.

2.6. Analysis

Effluent samples were passed through an ultrafree™ 0.22 µm centrifuge filter (Millipore, Bedford, MA) spun at 10 000 ×*g* and a sub-sample analyzed for atrazine, and hydroxyatrazine with an HPLC instrument (Shimadzu LC, Shimadzu Scientific Instruments, Inc. Columbia, MD) equipped with a photodiode array detector (SPD M 10A detector, Shimadzu Scientific Instruments, Inc. Columbia, MD). Analytes were separated on a C_{18} column (Econosphere C_{18} column 5 µm and 150×4.6 mm, Alltech Associates, Inc, Deerfield, IL). The mobile phase was acetonitrile:5 mM ammonium acetate adjusted to pH 4.5 (35:65 v/v) and was run isocratically at 40 °C at a flow of 1 mL min^{-1}. The injection volume was 100 µL. Atrazine was detected at 223 nm and the retention time was 8.7 min. Hydroxyatrazine was detected at 236 nm and the retention time was 4.65 min. The limit of detection was 5 ng mL^{-1} (n=8). Nitrate was estimated via a high-pressure liquid chromatographic procedure (Hunter et al., 1997).

2.7. Extraction of column segments

At the end of the initial study the sand from each of the biobarrier containing columns was collected in 5 cm segments and samples of this material (5 g) weighed into 50-mL centrifuge tubes and extracted with 15 mL 80:20 (v/v) MeOH/25 mM ammonium acetate adjusted to pH 8.0.

The suspension was agitated on a horizontal shaker for 30 min, centrifuged at 8000 ×*g* for 15 min, and the supernatant transferred to 50-mL centrifuge tubes. The extraction procedure was repeated and supernatants combined. Supernatant was then evaporated to less than 5 mL at 50 °C with a Rapidvap, brought to 10 mL with deionized water, and concentrated on a C_{18} SPE column (Thermo Electron Corp Hypersep) preconditioned with 3 mL each of methanol, ethyl acetate, methanol, and distilled water. The column was dried under negative pressure for 90 min, and atrazine, deethylatrazine, and deisopropylatrazine were eluted with 2 mL ethyl acetate into 2-mL volumetric tubes. Samples were fortified with an internal standard, 10 µL of 0.1 mg mL^{-1} of butylate dissolved in acetonitrile, brought to volume with ethyl acetate, and analyzed by GC/MS (described below). Subsequently, hydroxyatrazine was eluted from the column with 2 mL 95:5 (v/v) methanol/0.1 N HCl into 2 mL volumetric tube. Samples were brought to volume with methanol and analyzed by HPLC as described above.

2.8. GC/MS analysis

The parent compound and *N*-dealkylated metabolites were quantified by monitoring the masses of atrazine (*M*/*Z* 200), deethylatrazine (*M*/*Z* 172), deisopropylatrazine (*M*/*Z* 173), and butylate (*M*/*Z* 146) with a gas chromatograph (GC) equipped with a mass spectrometer (MS) (Shimadzu GC-17A and GC-MS QO 5050A, Shimadzu Scientific Instruments, Columbia, MD). Analyte separation was achieved on a 30-m by 0.25-mm RTZ-5 column (Restek, Bellefonte, PA) with a flow of helium at 1 mL min^{-1}. Injection and detector temperature were held at 280 °C. Initial oven temperature was held at 80 °C for 1 min, ramped to 250 °C at 20 °C min^{-1}, and held for 1.5 min. Total run time was 11 min. Under these conditions the retention times of butylate, deisopropylatrazine, deethylatrazine, and atrazine were 6.51, 7.89, 7.96, and 8.44 min, respectively. Recovery of atrazine, deisopropylatrazine, and deethylatrazine from fortified samples (n=8) was 95, 85, and 90%, respectively. The method limit of quantitation for atrazine, deisopropylatrazine and desethylatrazine was 0.005 mg kg^{-1}.

2.9. Partitioning of atrazine and N-dealkylated metabolites into soybean oil

The partitioning of the parent compound and *N*-dealkylated metabolites into soybean oil was quantified by placing 10 mL of soybean oil plus 10 mL of HPLC-grade water into a 50 mL glass tube. To these tubes 10 µg of atrazine, deethylatrazine, deisopropylatrazine or hydroxyatrazine was added and the tube capped with a Teflon-lined lid. The tubes were shaken horizontally for 16 h and then centrifuged at 2000 ×*g* for 15 min. Three milliliters of the water phase was transferred to a 7 mL glass tube, 3 mL of water-saturated toluene was added, and the tubes shaken for 30 min. The tubes were then centrifuged at 2000 ×*g* for 15 min and the amount of atrazine, deethylatrazine or deisopropylatrazine was quantified by GC/MS as described previously. The level of hydroxyatrazine in the water phase was determined by filtering 700 µL of the water phase through a 0.22 um filter and analyzing by HPLC as described previously. The measurements were replicated 4 times. The soybean oil:water partitioning coefficient was calculated as:

(µg of compound/mL soybean oil)/(µg of compound/mL water)

3. Results

3.1. Initial investigation

An initial investigation showed that the amount of atrazine in a simulated groundwater was significantly reduced as it was pumped through the biobarriers (Fig. 2B). During the first 30 weeks of the study ~5.8 L (85 column pore volumes) of water containing ~1 mg L^{-1} atrazine was pumped through each of the biobarriers at flow rates of ~28 mL day^{-1} (0.41 column pore volumes per day). Atrazine was introduced into the system at the start of the study and its presence in the effluent water increased for the first several weeks of the study. During the 5th week of the study an atrazine-degrading soil inoculum was applied to the columns and on week 7, 2 weeks after the addition of the bacterial inoculum, atrazine levels in the column effluents begin to drop (Fig. 2B). This decline continued until the 24th week of the study when

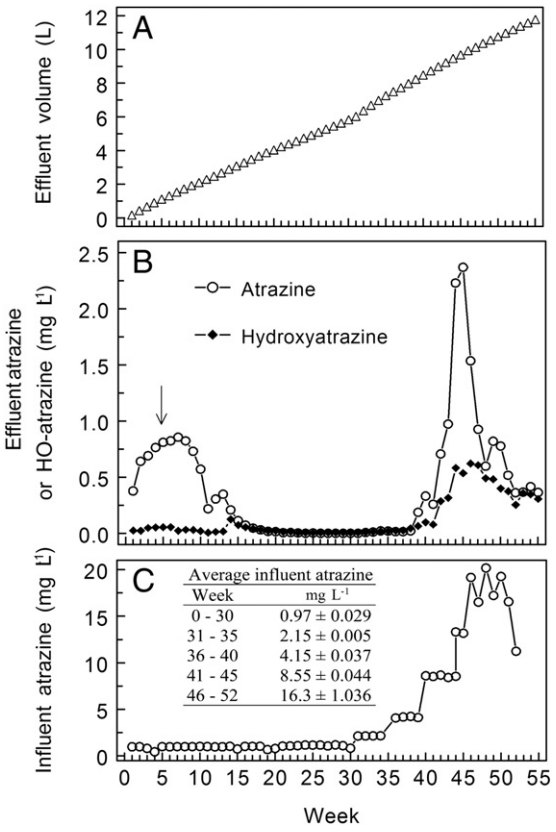

Fig. 2. Atrazine and hydroxyatrazine concentrations in the effluents of sand columns containing vegetable oil-based biobarriers. Cumulative volume of effluent water collected (A), concentration of atrazine in the effluent water (B), and concentration of atrazine in the influent water supplied to the columns (C). An inoculum of atrazine-degrading soil bacteria was injected onto the columns during the 5th week of the study (arrow). Each data point is an average of data obtained from two independent columns.

atrazine was no longer detected (LOD=0.005 mg L^{-1}) in the effluent waters.

Column effluents were assayed for hydroxyatrazine and small amounts were detected. During the first 30 weeks of the study the amount of hydroxyatrazine in the effluents averaged 0.028±0.004 mg L^{-1} (Fig. 2B).

The first phase of this study presents evidence that under the appropriate conditions vegetable oil-based biobarriers can be used to remove 1 mg L^{-1} atrazine from contaminated groundwater. Using the same biobarrier containing columns we continued this study into a second phase where we examined the ability of biobarriers to remove larger amounts of atrazine from flowing groundwater. We initiated this phase of the study on week 31 by increasing the level of atrazine in the influent water to ~2 mg L^{-1}. This was followed by additional stepwise doublings of the amount of atrazine in the influent water over the following weeks of the study until the influent water contained ~16 mg L^{-1} atrazine (Fig. 2C). Analysis of the effluents showed that the columns were able to degrade most but not all of the increased amounts of atrazine. During week 45, when influent atrazine levels were at 8.55 mg L^{-1}, atrazine levels in the effluents peaked at 2.37 mg L^{-1}. However, this increase in effluent atrazine level was brief and effluent atrazine levels declined to below 0.5 mg L^{-1} in the following weeks even as we increased the influent atrazine level to ~16 mg L^{-1}. In the final weeks of the study the amount of atrazine in the effluent water was steadily declining and it is possible that the amount of atrazine in the effluent water would have declined farther had we continued the study. Hydroxyatrazine levels in the effluents also increased during this phase of the study; hydroxyatrazine levels peaked at 0.62 mg L^{-1} on the 46th week of the study and averaged 0.38±0.04 mg L^{-1} during weeks 40 through 55.

3.2. Determination of the amount of atrazine or hydroxyatrazine sequestered by the sand columns

Atrazine partitions into soybean oil (Table 1) but there was no evidence that atrazine or hydroxyatrazine accumulated in large amounts in the oil or other components of the columns during the study. At the end of the study the columns from the above study were disassembled into segments and the sand matrix from the columns assayed for the presence of atrazine, hydroxyatrazine, deisopropylatrazine and desethylatrazine (Fig. 3). During the study ~44 mg of atrazine was applied to each column. However, of this amount only 0.025 mg of atrazine, 0.06% of that applied, was recovered from the sand and oil within the columns indicating that

Table 1
Partitioning of atrazine and several atrazine degradation products between soybean oil and aqueous 0.02 M $CaCl_2$

Herbicide	Partition ratio (Soybean oil:aqueous)
Atrazine	61:39
Deethylatrazine	3:97
Deisopropylatrazine	1:99
Hydroxyatrazine	0:100

Analysis was by GC-MS.

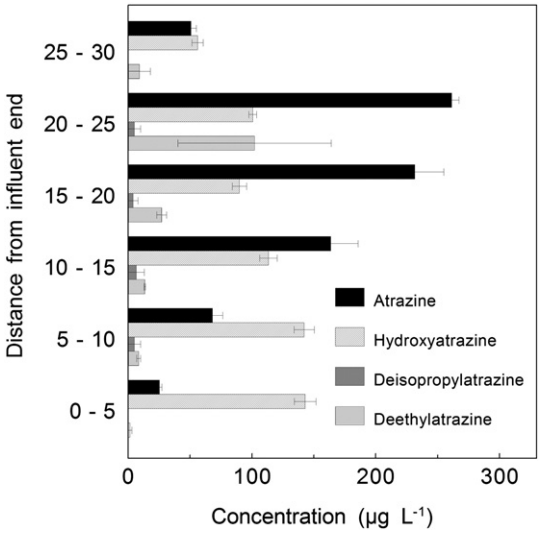

Fig. 3. Atrazine and hydroxyatrazine sequestered in column segments.

atrazine did not sequester in the biobarriers inside of the columns during the study. Amounts of hydroxyatrazine extracted from the sand and oil from within the columns was slightly lower than that seen with atrazine and accounted for about 0.05% of the atrazine applied to the columns during the study. Deisopropylatrazine and deethylatrazine were also detected but in even smaller amounts. These results show that these compounds did not accumulate within the columns in large amounts. Hydroxyatrazine, deethylatrazine and deisopropylatrazine do not absorb into vegetable oil in significant amounts thus losses due to the partitioning and sequestering of these atrazine degradation products into the vegetable oil substrate would be expected to be minor (Table 1).

3.3. Atrazine removal in the presence of nitrate

The presence of nitrate in the influent water may influence the efficiency of microbial based biobarriers used for atrazine remediation. This is because atrazine can serve as a source of nitrogen when other sources of nitrogen are limiting (Radosevich et al., 1995) and bacteria that can degrade atrazine are favored in such an environment. However, when nitrate is available as a nitrogen source atrazine-degrading bacteria may switch to nitrate (Gebendinger and Radosevich, 1999) or, alternatively, non-atrazine-degrading bacteria may be favored in such an environment. For these reasons a study that evaluated the ability of vegetable oil-based biobarriers to remove atrazine from groundwater when the water supplied to the biobarriers contained both atrazine and nitrate. The results showed that the presence of 5 mg L^{-1} nitrate-N in the influent water reduced but did not completely block the ability of the biobarriers to remove atrazine from water (Fig. 4). Whereas, in the initial study using columns that contained no nitrate, the vegetable oil-based biobarriers achieved a removal efficiency of 100%, in this study removal efficiencies averaged about 41% during the final 35 weeks of the study and the best removal efficiencies achieved were 49% for a period between weeks 35 and 40 and

of 52% in the final 4 weeks of the study. As has been shown by earlier studies, barriers of this type were highly effective at removing nitrate from the influent water (Hunter et al., 1997). Nitrate levels in the effluent waters from the two columns in this study averaged less than 0.03 mg L^{-1}. Nonetheless, the presence of nitrate-N in the influent water clearly interfered with the ability of the biobarriers to remove atrazine.

3.4. Biological vs. abiotic degradation

Is the degradation of atrazine in these biobarriers due to biological activity or is its disappearance due to non-biological factors? In order to answer this question a study using mercury poisoning was conducted. The setup for this study was identical to that of the initial study except that on the 32nd week of the study the biobarrier containing columns were poisoned with 0.05 mM HgCl. Prior to the HgCl addition these column biobarriers behaved similar to those in the earlier study though the efficiency of atrazine removal was not quite as good. With these biobarriers atrazine levels in the effluents increased to equilibrate at about 1 mg L^{-1} as water containing 1 mg L^{-1} atrazine was pumped through the barriers (Fig. 5). This increase in atrazine levels reversed and atrazine levels began a slow decline following the addition of an atrazine-degrading bacterial inoculum; and by the 32nd week of the study, when the HgCl was added, atrazine levels in the effluents had declined to 0.031 mg L^{-1}, a 97% removal efficiency. Following the addition of the HgCl to the columns atrazine levels in the effluent increased rapidly to 0.74 mg L^{-1}. This rapid rise in the amount of atrazine in the biobarrier effluents suggest that biological degradation was the principal

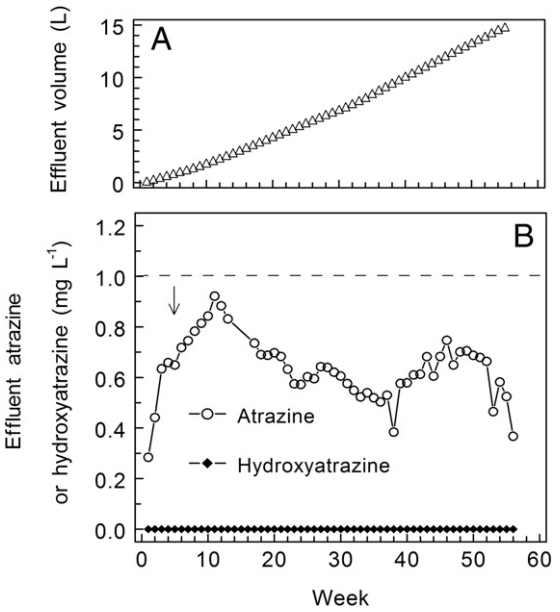

Fig. 4. Influence of nitrate on atrazine and hydroxyatrazine concentrations in biobarrier effluents. Cumulative volume of effluent water collected (A) and concentration of atrazine in the effluent water (B). Dashed line indicates average influent atrazine concentration. An inoculum of atrazine-degrading soil bacteria was injected onto the columns during the 5th week of the study (arrow). Each data point is an average of data obtained from two independent columns.

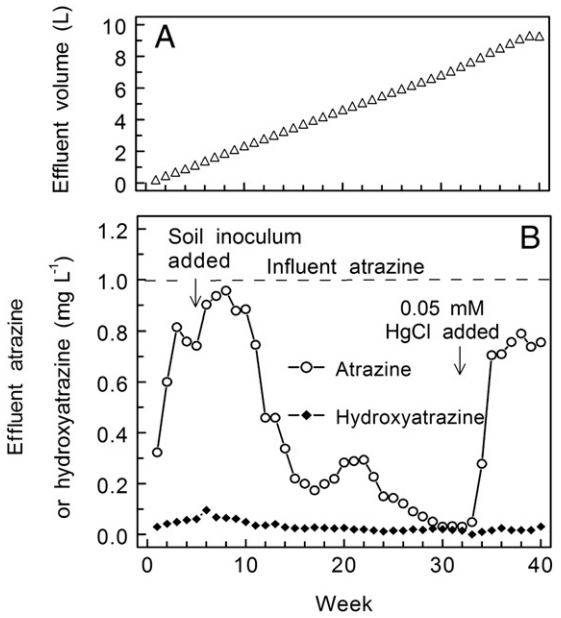

Fig. 5. Influence of mercury poisoning on atrazine and hydroxyatrazine concentrations in biobarrier effluents. Cumulative volume of effluent water collected (A) and concentration of atrazine in the effluent water (B). Dashed line indicates average influent atrazine concentration. An inoculum of atrazine-degrading soil bacteria was injected onto the columns during the 5th week of the study and HgCl was added to the influent water on the 32nd week of the study (arrows). Each data point is an average of data obtained from two independent columns.

activity responsible for the degradation of atrazine as it was pumped through the barriers.

4. Discussion

The results show that vegetable oil-based biobarriers can efficiently remove the atrazine parent compound from flowing water and can do so without the accumulation of toxic degradation products. This study supports the hypothesis that such barriers might serve as a useful tool for the remediation of atrazine contaminated aquifers or for the protection of aquifers from atrazine spills.

In most soils biological degradation is thought to be the principal method by which atrazine is decomposed. It may proceed by dehalogenation leading to the formation of hydroxyatrazine or via dealkylation resulting in the formation of deethylatrazine or deisopropylatrazine (Kaufman and Kearney, 1970; Barriuso and Houot, 1996; Abdelhafid et al., 2000a,b). In low pH soils, abiotic chemical dehalogenation can be the principal means by which atrazine is degraded (Da Silva et al., 2000; Mahía and Díaz-Raviña, 2007). In our biobarriers the primary method by which atrazine was removed was by microbial degradation of the parent compound. Several lines of evidence support this conclusion: 1) The amount of atrazine in the biobarrier effluents increased in the weeks preceding the application of the atrazine-degrading biological inoculum and declined after the inoculum was applied to the biobarriers. 2) Nitrate inhibition of atrazine degradation suggests biological involvement. 3) The addition of mercuric chloride inhibited the degradation of

atrazine by ~74%. Mercury is a broad spectrum antimicrobial agent that is effective against both prokaryotic and eukaryotic microorganisms and is often used to distinguish between biotic and abiotic activities. While its mechanism of action is unclear for bacteria, mercury interferes with transcription in archaea and in eukaryotes (Dixit et al., 2004). Inhibition by mercuric chloride indicates that biological processes were the principle processes involved in the degradation of atrazine. We made no attempt to determine if oxygen was involved in the degradation process but we expect that it was the primary electron acceptor. While much of the biobarrier may have been in an anaerobic state (Hunter et al., 1997) the influent water was fully oxygenated and we would expect that the front part of the biobarrier was aerobic.

The initial products of microbial degradation may accumulate or the degradation may continue without the accumulation of intermediates until only carbon dioxide and ammonia remain. We assayed the column effluents for atrazine and its degradation product hydroxyatrazine. Hydroxyatrazine was detected in the effluents indicating that microbial dehalogenation was one of the pathways of atrazine degradation used by the microbial consortium within the column biobarriers but does not rule out the possibility that dealkylation pathways may also have been operating. Dehalogenation abolishes the toxicity of the degradation products while dealkylation does not (Kaufman and Blake, 1970). Thus, hydroxyatrazine is relatively innocuous, it has no known toxic effects on plants or animals (Clay and Koskinen, 1990), and its presence in the effluent waters is of much less concern than would be the presence of atrazine, deisopropylatrazine or deethylatrazine.

Respiratory oxidation–reduction reactions are often the driving mechanisms behind in-situ bioremediations that involve the introduction of a carbon substrate. Electron donor availability usually limits microbial activity in deeper soils and aquifers (Myrold and Tiedje, 1985; Starr and Gillham, 1993) and microbial growth and respiration can be greatly stimulated by the addition of compounds (organic carbons, sulfur, etc.) that can function as a respiratory electron donor. This increase in respiration drives the system anaerobic and groundwater contaminants that can serve as microbial respiratory electron acceptors (i.e. nitrate, perchlorate, trichloroethylene, etc.) are often reduced to less harmful compounds in such environments. In contrast, the microbial degradation of atrazine involves its potential as a microbial nitrogen source rather than as a respiratory electron acceptor and the environment that needs to be established within the biobarrier to stimulate the degradation of atrazine must be one where it is the supply of nitrogen that limits microbial activity. Other growth requirements including the electron donor(s) and electron acceptor(s) should be available in excess. Since nitrogen will interfere it is important that the carbon substrate used to simulate its degradation have a low nitrogen content. This is why nitrate-N at 5 mg L^{-1} interfered with the ability of our biobarriers to remove atrazine. This interference was expected as several earlier investigators have shown that the microbial degradation of atrazine is slowed by the presence of nitrogen amendments such as nitrate. This was true both with soil studies using the native populations (Entry et al., 1993; Alvey and Crowley, 1995; Abdelhafid et al., 2000a,b) and with studies using axenic cultures (Radosevich et al., 1995; Struthers et al., 1998; Gebendinger and Radosevich, 1999; García-González et al., 2003). García-González et al. (2003) studied atrazine degradation by *Pseudomonas* sp. strain ADP and concluded that the degradation pathway was repressed when the nitrogen sources that were available permitted a high growth rate and induced when the available nitrogen sources slowed growth. Induction of the pathway did not require that atrazine be present; instead, the intracellular nitrogen status of the organism controlled atrazine degradation. Nitrate and atrazine would often occur together in groundwaters impacted by non-point source agricultural pollution and the interference caused by nitrate would need to be considered in any remediation project utilizing biological degradation. To overcome this problem it has been suggested that Nas^{-} mutants which are deficient in nitrate assimilation and therefore degrade atrazine when nitrate is present might be used to remediate soils that are contaminated with atrazine (García-González et al., 2003). The use of Nas^{-} mutants has not been evaluated in vegetable oil-based biobarriers. Other bacteria might also be used to inoculate biobarriers as well. The atrazine-mineralizing bacterium M91-3 is capable of degrading atrazine in the presence of nitrate as well as under aerobic, microaerophilic and anaerobic conditions (Crawford et al., 2000). The ability of any introduced bacteria to compete and function under environmental conditions would need to be determined.

5. Conclusion

- This study supports the hypothesis that vegetable oil-based biobarriers might be used in the field to remove atrazine from contaminated aerobic aquifers or to protect aquifers from contamination from atrazine spills. Electron donors other than vegetable oil would also likely work but were not evaluated in this study. Within the barriers the principal method by which atrazine was removed was by microbial degradation and not by abiotic processes. In order for the biobarriers to work an active population of atrazine-degrading microorganisms was required.
- The mechanism used to stimulate the microbial degradation of atrazine differed from that normally employed in biobarriers. Often it is microbial respiratory processes that are involved in the removal of contaminants from groundwater. However, atrazine was degraded as a source of nitrogen and the environment within the biobarrier that was required to achieve this degradation was one in which it was nitrogen that limited microbial activity. Other growth requirements (nutrients, electron donors, and electron acceptors) must be present in adequate or excess amounts.
- Nitrogen limiting biobarriers might also be used to cleanse other nitrogen containing contaminants from groundwater. The contaminant would not need to function as a microbial electron acceptor or donor.
- Nitrate, at concentrations of 5 mg L^{-1} N, reduced the efficiency of the barriers. This interference by nitrate might present a problem under some agricultural situations as nitrate and atrazine are both common groundwater contaminants that often occur together when agricultural activities are the source of the groundwater contamination. A solution to this interference by nitrate is needed and will

be the subject of future research. Nonetheless, the method shows promise as a means of protecting groundwaters from atrazine contamination when nitrate concentrations are low. Barriers of this type might be especially useful as a means of remediating spills involving atrazine or related compounds.

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